EXPRESSION OF RECOMBINANT HUMAN BCHE IN THE MILK OF TRANSGENIC MICE

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ABSTRACT

Human butyrylcholinesterase (huBChE) has been successfully used as prophylaxis in animal models to prevent intoxication as a result of exposure to Organophosphate (OP) agents. Since large quantities of enzyme would be required for prophylaxis or treatment, we explored the production of recombinant huBChE (rc-huBChE) in the milk of transgenic animals. A DNA expression vector containing the huBChE cDNA under the control of the goat ß-casein promoter was used to generate transgenic mice lines. Milk collected from eight female mice contained active rc-huBChE at concentrations ranging from 0.15 to 1.8 g/L. The majority of the protein produced was found in the dimer form. *In vitro* nerve agent inhibition experiments demonstrated that rc-huBChE is similar to its human plasma counterpart in terms of broad-spectrum properties. The data from the expression of rc-huBChE in the milk of transgenic mice support the hypothesis that the mammary gland bioreactor can be used as a platform for the production of large quantities of active rc-huBChE.

1. INTRODUCTION

Human serum butyrylcholinesterase (huBChE) (EC 3.1.1.8) is a globular, tetrameric molecule with a molecular mass of approximately 340 kDa. Nine Asn-linked carbohydrate chains are found on each 574-amino acid monomeric subunit [1]. The tetrameric form of huBChE is the most stable and is preferred for therapeutic purposes [2]. Although the physiological function of huBChE is largely elusive, it is well established that it can combine with OP nerve agents and insecticides, and hydrolyze many ester-containing drugs such as cocaine and succinylcholine [3]. Therefore, huBChE may serve as a natural OP scavenger in vivo [4]. The OP compounds, belonging to anticholinesterase agents, were first developed in the 1930's to be used as insecticides. Their potency was recognized during World War II, and they were developed as nerve agents to use in chemical warfare [5]. Furthermore, OP and other anticholinesterase agents are extensively used as pesticides and pose a substantial occupational and environmental risk [6].

Administration of exogenous huBChE to increase the potential of the native BChE present in the circulation to scavenge anticholinergic agents is an effective and safe alternative treatment for the prevention of anti-AChE toxicity, in addition to the traditional treatment regimes of atropine (ACh-receptor antagonist) and oximes (reactivators) of the OP-modified AChE [7]. These conventional treatments rarely alleviate post-exposure toxicity although successfully decrease anticholinesterase-induced lethality. In contrast, it has been shown that a single pretreatment injection of plasma-derived huBChE allowed protection against a challenge of OPs in a variety of rodent models [8-10] and primates [11, 12]. A large quantity of material is required to neutralize OPs due to the 1:1 stoichiometry. Since a limited supply can be produced from expired human plasma (fraction VI) efforts have been directed

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towards expressing rc-huBChE in Xenopus oocytes [13], mammalian cell lines [2, 14], insects [15] and silk worms [16]. But these systems have had limited success so far.

It has been well documented that the mammary gland of transgenic animals is an alternative system for recombinant protein production [17]. A variety of recombinant human proteins have been expressed and secreted into the milk of transgenic animals [17, 18]. We report herein on the generation of transgenic mice expressing recombinant (rc-) huBChE in their milk and the initial characterization of the overexpressed protein. The produced rc-huBChE has similar properties to the plasma huBChE in terms of OP hydrolysis when tested *in vitro*.

2. MATERIALS AND METHODS

2.1 Plasmid Construction and Preparation of Transgene

The mammary gland specific expression vector was constructed using the human BChE cDNA sequence under the transcriptional control of the goat β-casein promoter followed by the β-casein secretion leader. The goat β-casein promoter, including 5' sequences up to exon 2, was amplified by PCR from genomic DNA isolated from goat blood. The 5.9 kb PCR product was subcloned into pUC18 vector (Promega, Madison, WI, USA). The resulting pUC18/bCN-1 vector was digested with XhoI and ligated with XhoI-digested 4.3 kb PCR product spanning exon 7 and the 3 ' end of the β-casein gene to generate pUC18/bCN vector. The human BChE cDNA was amplified by PCR from a cDNA clone (ATCC #65726) [19]. The PCR product was subcloned into pGEM-T easy vector (Promega, Madison, WI, USA) digested with XhoI, the BChE insert purified and ligated to XhoI-digested pUC18/bCN plasmid from above to generate pbCN-BChE. Linear DNA (Not I digestion), free of bacterial sequences, was prepared and used to generate transgenic mice by pronuclear microinjection.

2.2 Production of Transgenic Mice

The production and maintenance of transgenic mice were conducted at McIntyre Transgenic Core Facility of McGill University. Animal studies were carried out in accordance with the Guide for the Care and Use of Laboratory Animals as adopted by the U.S. National Institutes of Health. Transgenic mice were generated essentially as described in [20] in a FVB background strain (Charles River Laboratories, Wilmington, MA, USA). The transgene was microinjected into fertilized eggs, and 22 pups were born. At 2-3 weeks of age tail biopsies were taken, under anesthesia, and DNA was prepared according to standard procedures [21]. Transgenic founder mice were bred with wild-type mice of the same strain for the generation of F1, F2 and F3 generations.

2.3 PCR and Southern blot analysis

Transgenic mice were initially identified by PCR and subsequently confirmed by Southern blotting analysis. DNA content in samples was quantitated using fluorometer. PCR was performed by employing three sets of primers for each sample. Confirmation of intactness and copy number of the transgene was assessed using Southern blotting analysis. DNA (5μg) extracted from the tails of PCR positive animals were analysed by restriction enzyme digestion (HindIII), followed by gel electrophoresis, and transferred to a nylon membrane (Roche Diagnostics Canada, Laval, QC, Canada).

2.4 BChE activity assay and non-denaturing activity gel staining

Milk was collected (1-3 times) from mice during their 20-day lactation period. The amount of milk collected during each milking varied from 100 – 200 μl. The milk was analyzed for BChE activity using a BChE activity assay (Ellman assay) [22]. The assay was performed on whole milk, diluted with 0.1 M potassium phosphate buffer (pH 8.0) using a Cholinesterase (BTC) kit from Sigma Diagnostics as per supplier's protocol. One unit of enzyme activity was defined as the amount required to hydrolyze 1 μmol substrate per minute. A converting equation of 720 U = 1 mg purified human plasma BChE was used [23]. The identification of the oligomeric forms of rc-huBChE secreted by the mammary gland was

performed by analysis of milk samples using non-denaturing activity gels stained with 1 mM of butyrylthiocholine iodide according to the Karnovsky and Roots' method [24].

2.5 Western blot analysis

Proteins in diluted milk samples were separated by SDS-PAGE on a 4-20% Tris-Glycine polyacrylamide gel (pre-cast, Invitrogen, Burlington, ON, Canada) and electrophoretically transferred to an ECL nylon membrane (Amersham Biosciences, Baie d'Urfé, QC, Canada). After blocking of the unspecific binding with 5% nonfat milk in TBST [0.24% (w/v) Tris, 0.8% (w/v) NaCl, pH 7.6, 0.05% (v/v) Tween™ 20] the membranes were sequentially incubated with a polyclonal anti-human BChE antibody diluted at 1: 1,000 (Dako, Mississauga, ON, Canada) and with a HRP-conjugated secondary anti-rabbit IgG antibody, diluted at 1: 5,000 (Promega, Madison, WI, USA). Immunorecative bands were detected with the ECL chemiluminescence detection kit (Amersham Biosciences, Baie d'Urfé, QC, Canada), and analyzed by the FluorChem™ 8000 System (Alpha Innotech Corporation, San Leandro, CA, USA).

2.6 *In vitro* inhibition of rc-huBChE by OPs

Various amounts of nerve agent solutions (2 - 2,000 nM) were added to dilute mouse milk samples containing rc-huBChE collected from 3 F1 mice and incubated at 25°C for 5 min. Milk collected from a negative FVB mouse and treated in a similar way served as the negative control. Residual BChE activity (%) was measured by the Ellman assay using BTC as a substrate at 25°C. Data were analyzed and plotted by Microsoft Excel.

3. RESULTS

3.1 Identification of transgenic mice

The human BChE cDNA PCR amplified from a cDNA clone (ATCC #65726) [19] was inserted into a mammary gland expression cassette under the control of the goat ß-casein promoter and it was used to generate transgenic mice. Four F0 founders, 2 males and 2 females, were generated. Southern blot analysis revealed that 4 transgenic mice carried 8-10 copies of the huBChE transgene inserted in their genome. The integration frequency, i. e., transgenic mice /newborn animals, was 18%. Two transgenic founders, 43-3M and 61-2F, bred with wild type mice of the same strain, transmitted the gene construct to their offspring. About 50% of the F1 littermates were found to be transgenic. Thus, the transgene was transmitted according to the Mendelian rules of inheritance. Hemizygous mice of the two transgenic lines were viable and fertile.

3.2 Expression of rc-huBChE in the milk of transgenic mice

Milk samples collected from F0, F1, F2 transgenic mice 3-5 days after initiation of lactation were analyzed for the presence of rc-huBChE using non-denaturing polyacrylamide gels stained for cholinesterase activity [24], The rc-huBChE produced in the milk of the two high expressing lines 61-2B3F (**lane 2**) and 43-3A15F (**lane 3)** migrated as a mixture of dimer, tetramer and monomer with dimer being the predominant form (**Fig. 1**). The identity of the BChE oligomeric forms was confirmed further by using non-denaturing Western blot and a polyclonal anti-huBChE antibody (**Fig. 2**). The oligomeric form identification /assignment of rc-huBChE was based on migration relative to plasma huBChE (**lane 1** of **Fig. 1** and **2**, respectively). The results clearly demonstrate that rc-huBChE was produced and secreted by the mouse mammary gland. Furthermore, the overexpressed protein had no adverse effects in the lactation as transgenic females were able to nurse their pups with milk containing rc-huBChE at levels of up to 1.8 g/L (from 43-3A15F).

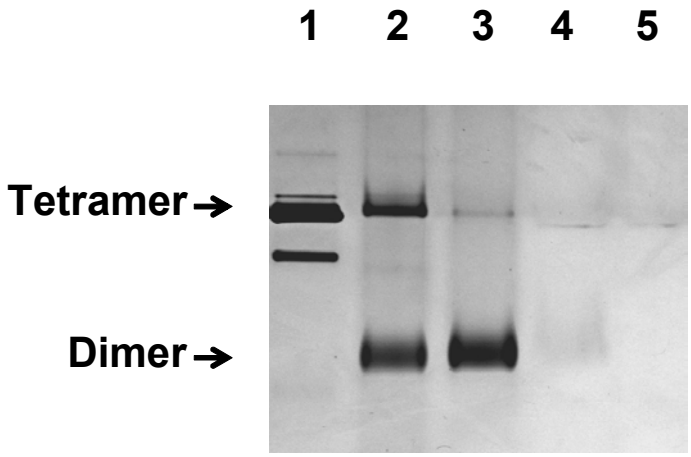

Fig. 1. Demonstration of rc-huBChE activity in milk of transgenic mice. Milk samples (10 µl) were loaded on native gels in the following order: **lane 1**, purified plasma huBChE (25 U/ml, courtesy of Dr. O. Lockridge); **lane 2**, diluted 61-2B3F milk (1:50); **lane 3**, diluted 43-3A-15F milk (1:1,000); **lane 4**, diluted 44-2 milk (1:3); **lane 5**, diluted FVB milk (1:3).

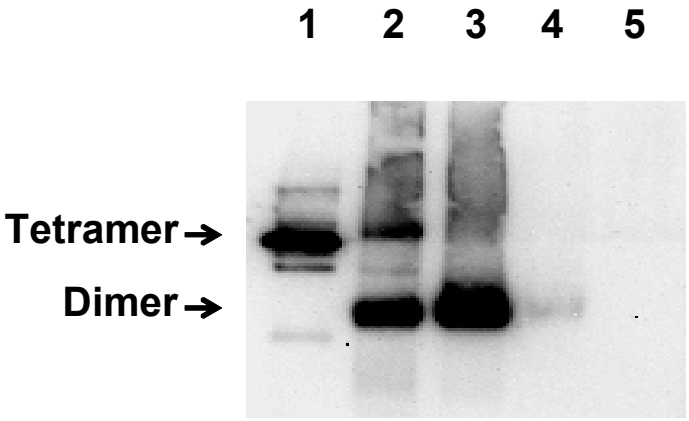

Fig. 2. Non-denaturing Western blot analysis. Immunodetection was performed with a polyclonal anti-huBChE antibody (Dako). 10 µl samples were loaded in the following order: **lane 1**, purified plasma huBChE (25 U/ml, courtesy of Dr. O. Lockridge); **lane 2**, diluted 61-2B3F milk (1:50); **lane 3**, diluted 43-3A-15F milk (1:1,000); **lane 4**, diluted 44-2 milk (1:3); **lane 5**, diluted FVB milk (1:3).

3.3 Inhibition of rc-huBChE by OP nerve agents *in vitro*

Milk samples collected from three transgenic mice as well as a non-transgenic mouse were incubated with various ratios with tabun, sarin, soman and VX. The results from this inhibition experiments are shown in **Fig. 3**. The amount of the nerve agents required to inhibit 100 % of enzyme activity was in good agreement with that of plasma huBChE published elsewhere [25], suggesting that rc-huBChE is similar to its plasma counterpart with comparable broad spectrum properties towards nerve agents.

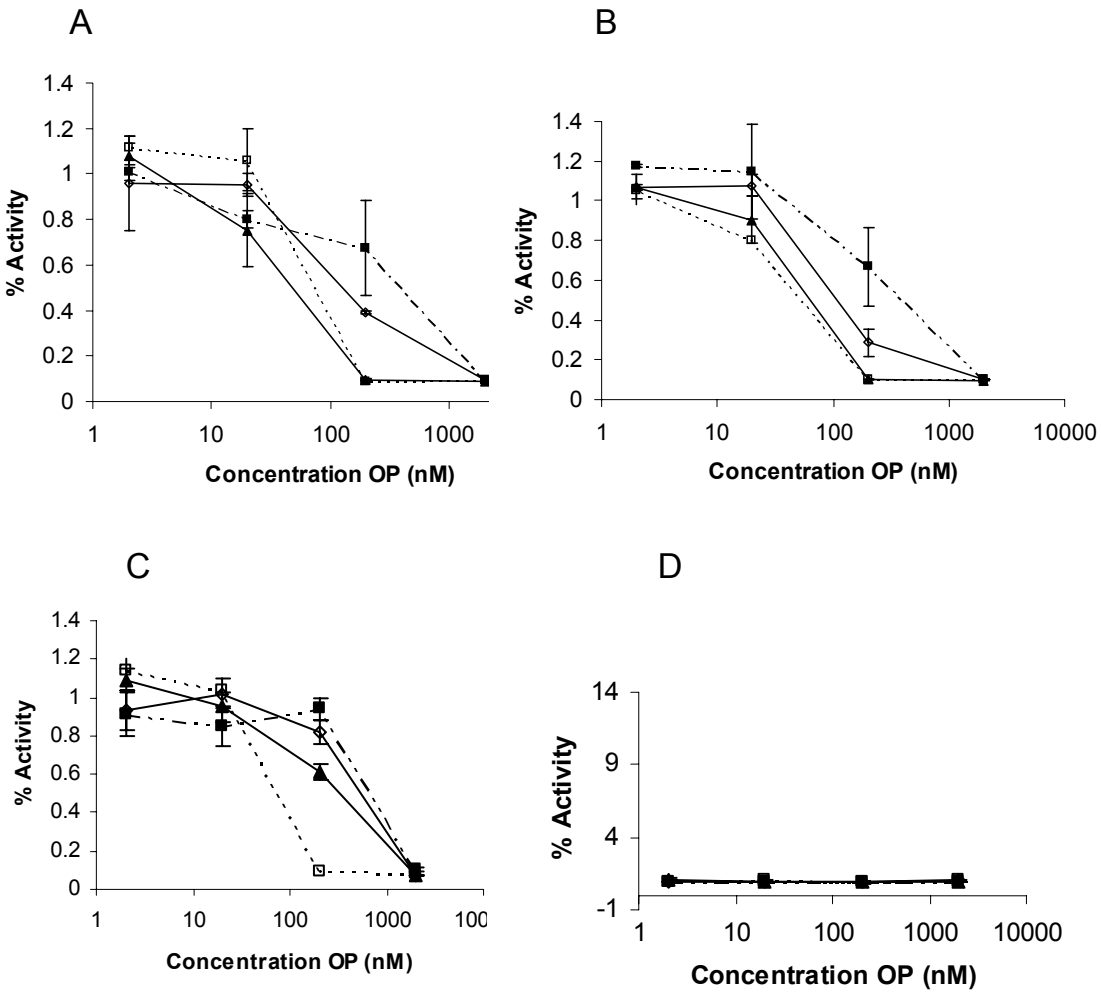

Fig. 3. *In vitro* binding and inhibition of rc-huBChE contained in the milk of transgenic mice by OP nerve agents. Binding and inhibition were performed with diluted raw mouse milk materials from three transgenic mice (**A**, **B** and **C**) and a control FVB (**D**). The reactions were carried in the presence of tabun (■), sarin (◊), soman (▲) and VX (□), respectively. Residual enzyme activity was measured by the Ellman assay.

4. DISCUSSION

4.1 High-level expression of rc-huBChE in the milk of transgenic mice

Plasma derived BChE can provide scavenger protection with a broad spectrum efficacy against nerve agents. This attribute has generated considerable interest in terms of structure and function related studies of the enzyme [3]. As a first step towards the feasibility of large-scale manufacturing of rc-huBChE in the milk of transgenic animals we have expressed rc-huBChE in transgenic mice, and have demonstrated the production of rc-huBChE in their milk at levels of 0.15-1.8 g/L.

4.2 Oligomeric form and glycosylation of rc-huBChE

It has been reported that the tetrameric form of huBChE is retained in the circulation for much longer period than some of its recombinant counterparts produced in cell culture systems [23]. The pharmaco-kinetic profile of the native enzyme in the circulation is mainly influenced by the oligomerization status of the enzyme as well as charge-and size-based heterogeneity in glycans [26]. Our non-denaturing activity gel staining analysis of the rc-huBChE established that the enzyme consists mainly of dimeric

form of BChE catalytic unit. The relative proportions of dimer, tetramer and monomer were approximately 60:30:10 whereas in human serum BChE, tetramer is the predominant form. It has been reported that ColQ PRAD peptide, a proline-rich attachment domain encoded by the ColQ gene, was shown to be necessary and sufficient for efficient assembly of tetrameric AChE [27]. This has also been demonstrated was proved later with recombinant BChE produced *in vitro* [23]. It is possible that the high-level of expression of rc-huBChE in the mouse mammary gland might have already exaggerated the BChE assembly machinery and the tetramer becomes a minor component of the total rc-huBChE.

Amongst all post-translational modifications, glycosylation is undoubtedly one of the most important aspects for therapeutic proteins [28]. The mammary gland cells have the capacity to secrete proteins with N-or O-glycosylated modifications. Glycans of the tetrameric form of human serum BChE, equine serum BChE and fetal bovine serum AChE have been reported to contain predominantly complex bi-antennary type of glycan structures which are responsible for the extended circulatory stability of the enzymes [7]. It would be essential to compare the circulation retention time of the rc-huBChE produced in the milk of transgenic mice with the native serum BChE.

4.3 rc-huBChE is functionally similar to plasma huBChE

For the milk expression system to be useful for manufacturing large quantities of rc-huBChE it is essential that the rc-huBChE is functionally similar to human BChE. *In vitro* nerve agent binding and inhibition experiments of rc-huBChE indicated that transgenically produced rc-huBChE was comparable to plasma huBChE [29].

5. CONCLUSIONS

The use of transgenic farm animals to produce pharmaceutically important recombinant proteins, such as antibodies, anti-clotting factors, and growth factors, in the mammary gland is well documented [18]. This technique enables the large-scale production of complex or unique molecules that might not be produced efficiently by any other method. In the studies presented herein, a DNA expression vector containing the huBChE cDNA under the control of the goat ß-casein promoter was established and used to generate transgenic mice. Milk collected from female mice contained active rc-huBChE at concentrations ranging from 0.15 to 1.8 g/L. The majority of the protein produced was found in the dimer form. *In vitro* nerve agent inhibition experiments indicated that rc-huBChE is similar to its human plasma counterpart. Therefore, the present study forms a solid base for producing large enough quantities of rc-huBChE in the milk of transgenic animals as an alternative and reliable source for BChE prophylaxis and treatment, in addition to the purification of human plasma BChE.

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